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Investigation of an Acetate-Fed Denitrifying Microbial Community by Stable Isotope Probing, Full-Cycle rRNA Analysis, and Fluorescent In Situ Hybridization-Microautoradiography

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The acetate-utilizing microbial consortium in a full-scale activated sludge process was investigated without prior enrichment using stable isotope probing (SIP). [^{13}C]acetate was used in SIP to label the DNA of the denitrifiers. The [^{13}C]DNA fraction that was extracted was subjected to a full-cycle rRNA analysis. The dominant 16S rRNA gene phylotypes in the ^{13}C library were closely related to the bacterial families *Comamonadaceae* and *Rhodocyclaceae* in the class *Betaproteobacteria*. Seven oligonucleotide probes for use in fluorescent in situ hybridization (FISH) were designed to specifically target these clones. Application of these probes to the sludge of a continuously fed denitrifying sequencing batch reactor (CFDSBR) operated for 16 days revealed that there was a significant positive correlation between the CFDSBR denitrification rate and the relative abundance of all probe-targeted bacteria in the CFDSBR community. FISH-microautoradiography demonstrated that the DEN581 and DEN124 probe-targeted cells that dominated the CFDSBR were capable of taking up [^{14}C]acetate under anoxic conditions. Initially, DEN444 and DEN1454 probe-targeted bacteria also dominated the CFDSBR biomass, but eventually DEN581 and DEN124 probe-targeted bacteria were the dominant bacterial groups. All probe-targeted bacteria assessed in this study were denitrifiers capable of utilizing acetate as a source of carbon. The rapid increase in the number of organisms positively correlated with the immediate increase in denitrification rates observed by plant operators when acetate is used as an external source of carbon to enhance denitrification. We suggest that the impact of bacteria on activated sludge subjected to intermittent acetate supplementation should be assessed prior to the widespread use of acetate in the wastewater industry to enhance denitrification.

Nonpolluted natural bodies of water, such as freshwater streams and coastal marine water, have been found to have limiting concentrations of both dissolved inorganic nitrogen and dissolved inorganic phosphorus (11). According to limiting-nutrient concepts, the growth of phytoplankton, macroalgae, seagrass, etc. is limited in these aquatic environments mainly due to the low availability of dissolved inorganic nitrogen and dissolved inorganic phosphorus. Dennison and Abal (11) demonstrated that stimulation of the growth of these organisms and a subsequent imbalance in the aquatic ecosystems is inevitable when dissolved inorganic nitrogen is not limiting in these bodies of water. Therefore, the control of nitrogen in bodies of water has been highlighted as an important environmental activity, and stringent standards for total nitrogen levels have been imposed for effluents released from wastewater treatment plants.

Biological nitrogen removal from wastewater is a two-step, sequential process. The first step is nitrification, an aerobic process in which ammonia is oxidized to nitrate (27). The second step is denitrification to remove soluble nitrate and nitrite from the wastewater (39). During denitrification facultative anaerobic microorganisms in activated sludge use nitrite or nitrate ions as final electron acceptors while utilizing organic matter as an electron donor and carbon source for growth (17, 19, 40). The limited soluble carbon in some treatment plants has resulted in difficulties in achieving the required effluent nitrogen levels, especially during periods when there are peak nitrogen loads (21, 24, 28, 43). Furthermore, for a given plant size and hydraulic load, low denitrification rates also result in unacceptable effluent nitrogen levels even when sufficient carbon is present for complete denitrification (30). The intermittent addition of readily biodegradable external carbon sources to the anoxic zones of treatment plants is an effective approach not only for supplementing the carbon deficiency for denitrification but also for achieving higher denitrification rates to meet effluent standards specifically during peak nitrogen load periods (25, 26, 28, 30, 43). A rapid increase in the denitrification capacity can result in reduced hydraulic retention times in the anoxic zones. This could facilitate the use of existing basin volumes in wastewater treatment plants to treat higher nitrogen loads.

According to Henze (29), the use of acetate and methanol results in denitrification rates that are three times higher than the rates for waste organic matter alone in raw wastewater. Although some reports (26, 43, 44) have demonstrated that acetate augmentation results in a higher rate of denitrification than methanol and ethanol augmentation, other reports (30) have suggested that sludge hydrolysate results in rates of denitrification similar to those observed with acetate.

The objective of this study was to use culture and largely enrichment-independent methods to identify denitrifiers from full-scale activated sludge that can utilize acetate as a sole source of carbon for denitrification. Therefore, stable isotope

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probing (SIP) with [^{13}C]acetate was used to directly label the DNA of denitrifiers present in full-scale sludge. No laboratory-scale enrichment was employed. The labeled DNA was used as a template for 16S rRNA gene amplification, and the amplificates were used for cloning, restriction fragment length polymorphism analysis, phylogenetic analysis, and fluorescent in situ hybridization (FISH) probe design. A laboratory-scale bioreactor enriched in acetate-utilizing denitrifiers was evaluated with the FISH probes. FISH-microautoradiography (MAR) using some of the FISH probes and [^{14}C]acetate confirmed that the probe-targeted bacteria were capable of taking up acetate under anoxic conditions. A combination of molecular methods was used to generate data that strongly suggest that bacteria related to the genera *Acidovorax*, *Dechloromonas*, and *Thauera* belonging to the families *Comamonadaceae* and *Rhodocyclaceae* are capable of utilizing acetate under anoxic conditions.

MATERIALS AND METHODS

Denitrification performance of Wacol sewage treatment plant biomass. A titration and off-gas analysis (TOGA) sensor (18) was adapted to study the denitrification properties of return activated sludge (RAS) from a full-scale biological nutrient removal sewage treatment plant (Wacol, Queensland, Australia). The plant exhibited consistent, high levels of nitrogen removal without a requirement for an external carbon source for denitrification. The TOGA sensor was used to maintain an in-reactor dissolved oxygen (DO) concentration of 0.00 mg liter^{-1} and a pH of 7.6 ± 0.01 by adding 1 M HCl and to measure the gas emission from the biomass in the bioreactor using a mass spectrometer (MS) (OmniStar; Balzers AG, Liechtenstein). The premixed gas used for MS calibration and for sparging through the bioreactor was composed of 2% N_2, 2% Ar, 0.49% CO_2, and 95.51% He (Linde Gas Pty. Ltd., Australia). An additional carrier gas (99.996% He; Linde) was used to dilute the reactor gas to a concentration range suitable for MS measurement.

RAS was collected from the Wacol sewage treatment plant on the same day that the analysis in the TOGA sensor was carried out and was diluted using treated effluent to obtain a mixed liquor volatile suspended solids (MLVSS) concentration of 3.8 g liter^{-1} (3). Three hundred milliliters of diluted Wacol sludge was placed in a 500-ml bioreactor which was connected to the TOGA sensor and was operated until a constant N_2 signal was obtained, which demonstrated that no denitrification was occurring. A mixture of 10 ml of 9.6-g liter^{-1} acetate and 10 ml of 15.86-g liter^{-1} $NaNO_2$ was injected into the bioreactor at the beginning of the TOGA sensor experiment. This introduced an excess of acetate for denitrification at a chemical oxygen demand (COD)/NO_2^--N ratio of 3.18:1, which is similar to the ratio used by Chen et al. (7). The acetate and $NaNO_2$ stock solutions were prepared using MilliQ water and were sterilized by autoclaving. Acetate and $NaNO_2$ were the only additives, while all the micro- and macronutrients needed for microbial metabolism were supplied by the sludge inoculum or treated effluent diluent. Gaseous nitrogen produced as a result of denitrification was measured continuously until the N_2 signal of the MS indicated that no further denitrification was occurring in the reactor.

Immediately after injection of the acetate-$NaNO_2$ mixture, 3-ml mixed liquor samples were collected from the bioreactor and filtered (Whatman nitrocellulose membrane; pore size, 0.2 μm) every 5 min for the first 0.5 h and then every 15 min until the end of the experiment. The samples collected were analyzed for ammonia, phosphate, nitrate, and nitrite by flow injection analysis (Lachat, Zellweger Analytical, Milwaukee, Wis.). Acetate in the filtered bioreactor contents was measured by high-performance liquid chromatography (Shimadzu Scientific Instruments, Inc., Columbia, Md.). At the end of the experiment, the MLVSS was measured. These TOGA sensor measurements and off-line analyses of freshly collected Wacol sludge were carried out on three different occasions to assess the reproducibility of the denitrification rates measured.

Microbiological analysis. (i) SIP of full-scale sludge. SIP was carried out with 300 ml of freshly diluted Wacol sludge (MLVSS concentration, 3.8 g liter^{-1}), which was placed in a 500-ml bioreactor connected to a TOGA sensor and exposed to [^{13}C]acetate (99% $^{13}CH_3$-$^{13}COOH$) for 48 h under strictly anoxic conditions. The experimental period was divided into eight cycles of 4 h of continuous feeding (acetate and NO_2^--N) and mixing, 1.5 h of mixing, 20 min of settling, 5 min of decanting the supernatant (225 ml), and 5 min of refilling with 200 ml treated effluent from the Wacol treatment plant. A COD/NO_2^--N ratio of 3.18:1 was maintained by adding 12.5 ml of a [^{13}C]acetate solution (10.8 g liter^{-1}) and 12.5 ml of an $NaNO_2$ solution (17.3 g liter^{-1}) at a flow rate of 0.1 ml min^{-1}.

The bioreactor was sampled (3 ml) three times during each of the eight cycles, at the beginning of the cycle just before continuous feeding was initiated, after 3 h, and just prior to settling. The samples were analyzed for ammonia, phosphate, nitrate, and nitrite as previously described.

(ii) DNA extraction, PCR and cloning of 16S rRNA genes, sequence data analysis, and probe design. DNA extraction and separation of the labeled denitrifiers were carried out using methods described by Ginige et al. (21). In brief, after 48 h of bioreactor operation with [^{13}C]acetate (eight 6-h cycles), the DNA from 10 ml of concentrated biomass was extracted. As a control, DNA from 10 ml of freshly collected concentrated Wacol sludge (i.e., sludge not fed with [^{13}C]acetate) was extracted. Both the DNA samples were separately subjected to density gradient centrifugation. Every 1 ml of DNA solution was mixed with 1 g of CsCl dissolved in Tris-EDTA buffer (pH 7.6). Ethidium bromide (0.8 ml of a 10-mg ml^{-1} solution per 10 ml of DNA-CsCl mixture) was added, the mixture was transferred to 5.1-ml Beckman quick-seal centrifuge tubes, and the DNA fractions were resolved by equilibrium centrifugation at 200,000 × *g* for 24 h at 20°C (21). Bands of ^{13}C-labeled DNA were collected from the gradient and purified using Microcon YM-10 centrifugal filter units (Millipore Corporation).

The purified [^{13}C]DNA fraction was subsequently subjected to full-cycle rRNA analysis using methods described previously (6, 21). Compilation of DNA sequences from the clone library, analysis of these sequences with BLAST (1), and phylogenetic analysis (evolutionary distance analysis and tests of robustness) to construct phylogenetic trees were conducted as described previously (9, 12).

Nearly full-length sequences from the ^{13}C-labeled clone library were used for probe design to specifically target groups of clone sequences (21). The oligonucleotides that were designed were synthesized and labeled at the 5′ end with the indocarbocyanine dye Cy3 (Thermohybaid Interactiva, Ulm, Germany).

(iii) Operation of a CFDSBR. A 1.8-liter Setric Genie laboratory fermentor operating as a continuously fed denitrifying sequencing batch reactor (CFDSBR) was seeded with RAS from the Wacol sewage treatment plant, and the contents were diluted with treated effluent to obtain an MLVSS concentration of 1.5 g liter^{-1}. The CFDSBR was operated under anoxic conditions for approximately 21 days at 22 ± 2°C with a 6-h cycle consisting of 4 h of continuous addition of 120 ml (0.5 ml min^{-1}) of a mineral base medium containing acetate and $NaNO_2$ (see below) and mixing, 1.5 h of only mixing, 20 min of settling, 5 min of supernatant decanting (1.36 liters), and 5 min of refilling with 1.3 liters treated effluent from the Wacol treatment plant. The hydraulic retention time was 10.8 h, and to maintain a sludge age of 7 days, 64 ml of mixed liquor was wasted in each cycle 5 min prior to the settling stage. Mixing was performed with an impeller at 200 rpm. The pH in the reactor was maintained at 7.6 ± 0.3 using a CO_2-N_2 gas mixture. To achieve the desired pH and anoxic conditions, the flow rate of each gas was regulated manually. Periodically the DO level in the mixed liquor was measured, but no DO was detected.

The medium base contained (per liter) 90 mg of $MgSO_4 \cdot 7H_2O$, 160 mg of $MgCl_2 \cdot 6H_2O$, 42 mg of $CaCl_2 \cdot 2H_2O$, 11.71 g of $NaNO_2$, 122 mg of Bacto peptone (Difco Laboratories, Detroit, Mich.), 20 mg of Bacto yeast extract (Difco Laboratories), 50 mg of NH_4Cl, 11.33 mg of KH_2PO_4, 25.67 mg of $Na_2HPO_4 \cdot 12H_2O$, 16.06 g of $CH_3COONa \cdot 3H_2O$, and 0.3 ml of a nutrient solution prepared as previously described (4). The medium was prepared with reverse-osmosis-deionized water and was sterilized by autoclaving. The COD/NO_2^--N ratio in the medium was maintained at 3.18:1.

Cycle studies were carried out every second day during operation of the CFDSBR. During these cycle studies, 25 ml of feed was "dump fed" at the beginning of the cycle instead of continuous feeding. Samples were collected every 5 min during the first 30 min and then every 15 min for 3 h of the 5.5-h anoxic reaction period. The NO_2^--N removal performance of the CFDSBR was assessed by comparing the supernatant nitrite concentration at the end of a cycle with the value for the influent. The samples were analyzed for ammonia, phosphate, nitrite, nitrate, and acetate using methods described previously. The MLVSS concentration in the reactor was also determined at the end of the cycle study.

(iv) Probe optimization community changes in CFDSBR and FISH-MAR. Due to a lack of pure cultures whose 16S rRNA bound most of the probes that were designed (Table 1) in this study, the optimum formamide concentrations for the probes were determined using fixed enriched CFDSBR sludge obtained on days 1, 3, 14, and 20 during enrichment. A similar approach of using mixed microbial biomass for probe optimization has been used previously (9, 14, 21, 42). Optimization was performed mainly using fixed reactor sludge samples as positive

TABLE 1. FISH oligonucleotides used in this study

Probe	Sequence (5′–3′)	rRNA target site[a]	Specificity	Formamide concn (%)	Reference
EUB338	GCTGCCTCCCGTAGGAGT	16S, 338–355	Most bacteria	20	2
EUB338-II	GCAGCCACCCGTAGGTGT	16S, 338–355	Bacterial groups not covered by EUB338 and EUB338-III	20	10
EUB338-III	GCTGCCACCCGTAGGTGT	16S, 338–355	Bacterial groups not covered by EUB338 and EUB338-II	20	10
BET42a	GCCTTCCCACTTCGTTT	23S, 1027–1043	*Betaproteobacteria*	35	36
GAM42a	GCCTTCCCACATCGTTT	23S, 1027–1043	*Gammaproteobacteria* (used as a competitor for BET42a)	35	36
DEN124	CGACATGGGCGCGTTCCGAT	16S, 124–143	Acetate-denitrifying cluster	40	This study
DEN220	GGCCGCTCCGTCCGC	16S, 220–234	Acetate-denitrifying cluster and some members of the *Comamonadaceae* family	40	This study
DEN220a	TCGGCCGCTCCGGAAGC	16S, 220–236	Acetate-denitrifying cluster and some members of the *Rhodocyclaceae* family	45	This study
DEN441	TGCGATTTCTTCCCGGCC	16S, 441–458	Acetate-denitrifying cluster and some members of the *Rhodocyclaceae* family	40	This study
DEN444	GAGAAGGCTTTTTCGTTCCG	16S, 444–463	Acetate-denitrifying cluster	40	This study
DEN581	TGTCTTACTAAACCGCCTGC	16S, 581–600	Acetate-denitrifying cluster	45	This study
DEN1454	CCGTGGCAATCGCCCCCC	16S, 1454–1471	Acetate-denitrifying cluster	40	This study
AT1458	GAATCTCACCGTGGTAAGCGC	16S, 1458–1478	*Azoarcus-Thauera* cluster in the *Betaproteobacteria*	50	41

[a] *E. coli* numbering for rRNA (5).

controls and fixed pure cultures as negative controls. The positive and negative controls that were used are described in Table 2.

Cell fixation and hybridization for FISH probe optimization were performed as previously reported (2, 4, 36). The probe optimization procedure was carried out as described by Ginige (20). In brief, the formamide concentrations for optimum stringency for the newly designed probes were determined by performing a series of FISH experiments with 5% formamide increments starting at 0% formamide. Probe dissociation was determined by plotting the cell probe intensity data with formamide concentrations in a scatter plot, and a sigmoidal curve was fitted to determine the probe dissociation formamide concentration. The dissociation curves for both the specific denitrifier probes designed in this study and the EUBMix probe with the target organism and the dissociation curves for the specific denitrifier probes with the nontarget organism were plotted on the same graph. Using the data of the three curves, an optimum formamide concentration (or an optimum range) for each specific probe was estimated. The formamide concentration that could be used for a specific probe was greater than the concentration (i) at which total dissociation of the specific probe with the nontarget organism was exhibited and (ii) at which the specific probe and EUBMix probe showed high fluorescence levels, which was just prior to these two probes reaching their dissociation levels against the target organism.

The shifts in microbial communities in the CFDSBR sludge were monitored and quantified every second day using previously described probes and probes that were designed in this study (Table 1). The methods used for microbial quantification have been described previously (20, 45).

Combined FISH and MAR were carried out with the enriched reactor sludge using methods described previously (21, 34). [^{14}C]acetate and probes DEN124 and DEN581 were used to assess the ability of the probe-targeted organisms to utilize acetate as a sole source of carbon under anoxic conditions.

RESULTS

Denitrification performance as determined with the TOGA sensor and by chemical analysis: Wacol RAS and biomass for SIP. The TOGA sensor indicated that there was an immediate response to the injected feed (acetate and nitrite), with instantaneous evolution of nitrogen gas from Wacol RAS. The rate of denitrification reached a maximum value of approximately 6 mg N_2-N mg MLVSS^{-1} h^{-1} during the 5-h anoxic period. Before the feed was introduced, the PO_4^{-3}-P concentration in the reactor was approximately 6.25 mg liter^{-1}, and after injection of the feed the PO_4^{-3}-P concentration increased to a maximum of 25.7 mg liter^{-1}. The NH_3-N concentration increased slightly from 0.87 mg liter^{-1} to 1.20 mg liter^{-1}. The NO_2^--N concentration, which was as high as 107 mg liter^{-1} initially, decreased to undetectable levels (<0.02 mg liter^{-1}) during the 5-h anoxic period.

During the first cycle of bioreactor operation for the SIP experiment, 92.4 mg liter^{-1} of NO_2^--N was denitrified from a total of 146.3 mg liter^{-1} that was introduced continuously into the reactor. During this cycle, the PO_4^{-3}-P concentration increased from 8.81 mg liter^{-1} to 22.91 mg liter^{-1}, and the NH_3-N concentration increased from 0.54 mg liter^{-1} to 1.77 mg liter^{-1}. From the fourth cycle onward, no nitrite accumulated in the reactor, and the biomass completely denitrified all the nitrite added (146.3 mg liter^{-1} NO_2^--N).

Full-cycle rRNA analysis. A single DNA band (^{12}C band) was observed in the control experiment in which DNA from freshly collected Wacol sludge was utilized. The sludge fed [^{13}C]acetate produced two bands upon centrifugation. One band was at the same position as the single ^{12}C band from

TABLE 2. Controls used for probe optimization

Probe	Negative control[a]	Positive control[b]	Negative control sequence[c]
DEN124	*Thauera aromatica* DSM 14793	Reactor sludge	---------U----------
DEN220	*Acidovorax avenae* subsp. *avenae* ATCC 19860	Reactor sludge	---A----------
DEN220a	*Propionivibrio dicarboxylicus* DSM 5885	Reactor sludge	----U----------
DEN441	*Thauera aromatica* DSM 14793	*Thauera linaloolentis* DSM 12138	----------UG
DEN444	*Variovorax paradoxus* ATCC 17713	Reactor sludge	----------CG--------
DEN581	*Methylophilus methylotrophus* ATCC 53528	Reactor sludge	----------C-----U--
DEN1454	*Ralstonia solanacearum* ACM 3851	Reactor sludge	----------A------

[a] Negative controls with less than three mismatches.
[b] The level of idenity with the probe was 100%.
[c] Only nucleotides which differ from the nucleotides in the probe sequence are shown.

TABLE 3. Clonal representation of microbial communities in the ^{13}C library

OTU	Accession no.	% of clones	Phylogenetic affiliation (closely related genus)
OTU-1	AY823958, AY823959	4	*Acidovorax* in the family *Comamonadaceae*
OTU-2	AY823960-AY823965	28	*Dechloromonas* in the family *Rhodocyclaceae*
OTU-3	AY823966-AY823968	9	*Thauera* in the family *Rhodocyclaceae*
OTU-4	AY823969, AY823970	9	*Thauera* in the family *Rhodocyclaceae*
OTU-5	AY823971-AY823973	14	*Dechloromonas* in the family *Rhodocyclaceae*
OTU-6	AY823974, AY823975	5	ND[a]
OTU-7	AY823976	5	*Thauera* in the family *Rhodocyclaceae*
OTU-8	AY823977, AY823978	4	*Dechloromonas* in the family *Rhodocyclaceae*

[a] ND, not determined.

Wacol sludge, and the other band was approximately 10 mm lower in the gradient. The latter band was assumed to be the band containing the ^{13}C-labeled DNA (^{13}C band). A total of 90 clones of the ^{13}C clone library (from ^{13}C-labeled DNA) was initially screened by restriction fragment length polymorphism analysis and assigned to 24 operational taxonomic units (OTUs), although the differences in banding patterns were often subtle (results not shown). Seven OTUs contained a total of 73 clones (22, 18, 16, 11, 2, 2, and 2 clones), while the remaining 17 OTUs contained one clone each. Partial sequencing of 57 clones representing 20 OTUs revealed that the clones were closely related to 16S rRNA gene sequences from organisms in the class *Betaproteobacteria*. The initial 24 OTUs were reorganized based on partial sequencing into eight main OTUs. Nearly complete sequences and phylogenetic analysis of 21 clones representing all eight OTUs revealed that the majority of these clones were relatives of the families *Comamonadaceae* and *Rhodocyclaceae*. These eight OTUs, their numerical representation in the clone library, and their phylogenetic affiliations with other organisms in the class *Betaproteobacteria* are shown in Table 3.

The nearly complete 16S rRNA gene sequences (positions 28 through 1491; *Escherichia coli* numbering) of the clones representing many of the OTUs mentioned above became the main focus for oligonucleotide probe design because (i) previously identified denitrifiers were found to be closely related to the families *Comamonadaceae* and *Rhodocyclaceae* (8, 33, 37, 41) and (ii) 78% of the clones in the clone library were found to be relatives of these two families. Seven oligonucleotide probes were designed; DEN220 and DEN444 targeted clones in OTU-1, DEN441 and DEN581 targeted clones in OTU-2, DEN124 targeted clones in OTU-3 and OTU-4, and DEN220a and DEN1454 targeted clones in OTU-5. The optimum formamide concentrations for the probes that were designed were determined from probe specificity and dissociation curves (Table 1).

Based on FISH images taken from hybridizations to CFDSBR biomass cells, the DEN220 and DEN444 probes targeted morphologically distinct organisms that formed chains of short, relatively thin rods (results obtained with DEN444 are shown in Fig. 1A). Many of the cells also had an internal vacuole-like structure (not shown). The DEN220 probe showed good binding to specific chains of short rods at a formamide concentration of 45% and failed to bind to its negative control, *Acidovorax avenae* subsp. *avenae*, which had a single base mismatch. At a formamide concentration of 40% in the FISH analysis, DEN444 clearly bound the chains of short rods in fixed sludge, while no binding of the probe was observed with the negative control *Variovorax paradoxus*, which had two base mismatches in the probe binding region.

DEN441 showed strong binding at a formamide concentration of 50% with its positive control, *Thauera linaloolentis*, but no hybridization with its negative control, *Thauera aromatica* (not shown). At a formamide concentration of 45% in the FISH analysis, the DEN581 probe exhibited strong binding to distinct cells in fixed sludge (Fig. 1B) and failed to hybridize with the negative control *Methylophilus methylotrophus*. At a formamide concentration of 40% in the FISH analysis, probe DEN124 strongly bound to specific organisms in the sludge but showed no hybridization to the negative control, *T. aromatica*. The DEN124-targeted cells had three distinct morphotypes (Fig. 1C): (i) short fat rods with a vacuole-like structure in the middle, (ii) comma-shaped bacteria resembling an open-ended coccus form that contained a vacuole-like structure, and (iii) cocci with a vacuole-like structure in the middle. The DEN124 targets were also easily observed because of their unique morphology and because of their high abundance in the CFDSBR biomass. The DEN220a and DEN1454 targets were also relatively thin short rods with either one or two internal vacuole-like structures, and they occasionally formed cell pairs (DEN1454-targeted cells are shown in Fig. 1D) at formamide concentrations of 40% and 45%, respectively. However at these formamide concentrations, the negative controls *Propionivibrio dicarboxylicus* and *Ralstonia solanacearum* failed to show any probe binding.

Phylogenetic analysis of the nearly complete sequence data for all OTUs demonstrated that all of the sequences obtained came from bacteria in the class *Betaproteobacteria*, which was confirmed by FISH of the CFDSBR biomass with probe BET42a (for *Betaproteobacteria*) and specific probes (data not shown). The probe-targeted cells exhibited no noteworthy clustering (Fig. 1) compared to the flocculated sludge that was used as the inoculum (data not shown).

CFDSBR: denitrification performance and microbial community analysis. The CFDSBR denitrification was monitored during biomass enrichment. On day 0 the nitrite removal efficiency was quite low, as only 4.85 mg liter^{-1} of the 24.1 mg liter^{-1} NO_2^--N that was introduced was removed. By day 3 a considerable improvement in the denitrification rate was evident, as all nitrite added to the reactor (24.1 mg liter^{-1}) was removed in 2.25 h during the anoxic reaction period. By day 6,

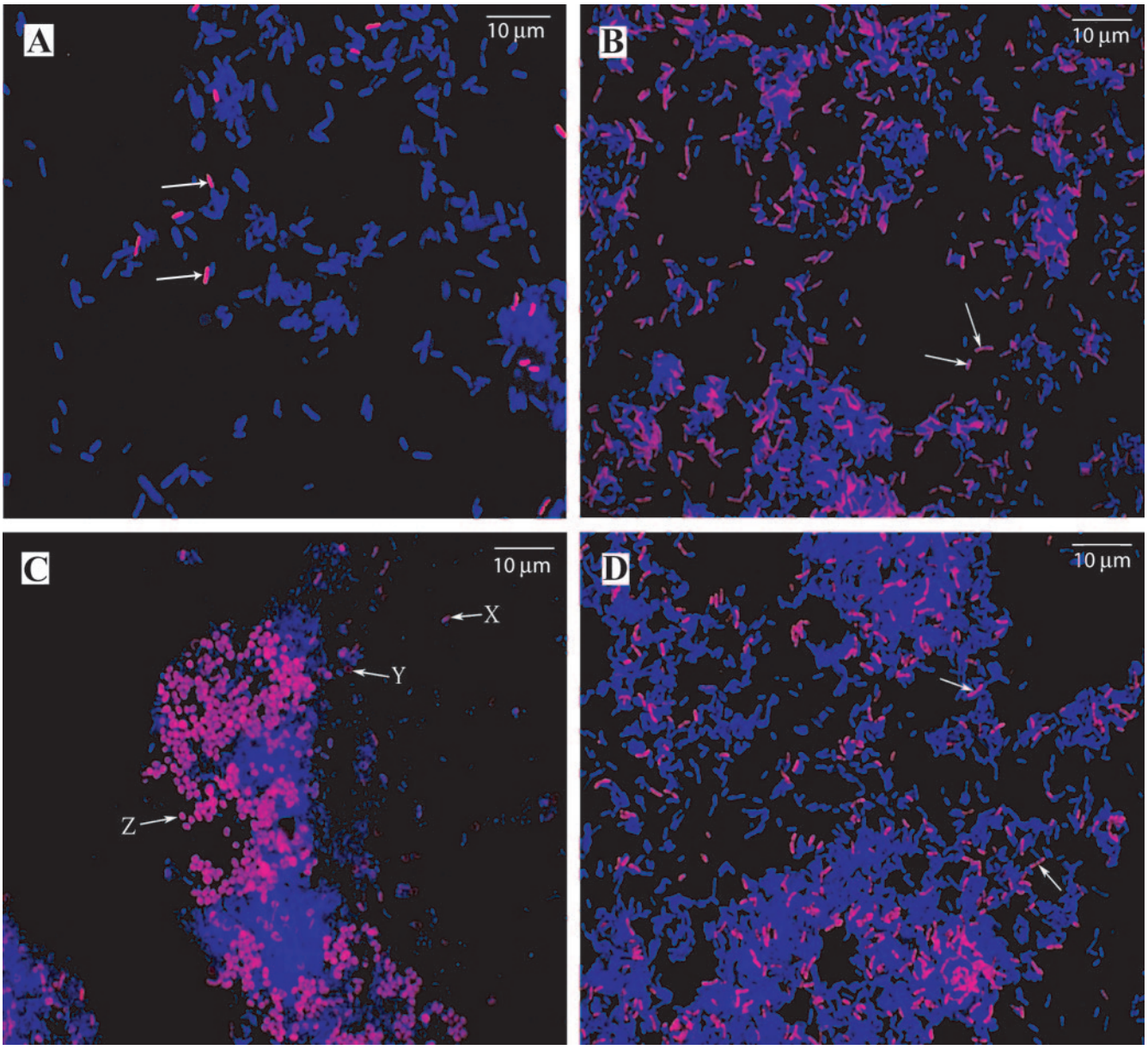

FIG. 1. Confocal laser scanning micrographs of CFDSBR biomass. Magenta cells are specific DEN probe-targeted bacteria, and blue cells are other bacteria. (A) Dual hybridization with the EUBMix and DEN444 probes. (B) Dual hybridization with the EUBMix and DEN1454 probes. The arrows indicate DEN1454-targeted cells. (C) Dual hybridization with the EUBMix and DEN581 probes. Arrow X indicates a short fat rod with a vacuole-like structure in the middle; arrow Y indicates a comma-shaped bacterium resembling an open-ended coccus form that contains a vacuole-like structure; and arrow Z indicates a coccus with a vacuole-like structure in the middle. (D) Dual hybridization with the EUBMix and DEN124 probes. The arrows indicate DEN124-targeted cells.

nitrite removal took only 55 min, and this rate was maintained throughout the experimental period (16 days).

Only the specific DEN444, DEN581, DEN124, and DEN1454 probes were used for detailed FISH quantification of the CFDSBR biomass; the broader probes, DEN220, DEN441, AT1458, and DEN220a, were not used. These probes and previously described phylogenetic-group-specific probes (Table 1) revealed the microbial community shifts in the CFDSBR with changes in the specific denitrification rates (Fig. 2). Initially, the levels of the probe DEN444-, DEN581-, DEN124-, and DEN1454-targeted microorganisms were relatively low, and these organisms comprised just 2%, 5%, 2%, and 4%, respectively, of the bacteria in the CFDSBR. However, after 6 days the levels of these organisms rose to 10%, 29%, 26%, and 22%, respectively, and they collectively comprised 87% of all EUBmix-targeted bacteria. In this period, the denitrification rate in the reactor increased from 0.001 to 0.063 mg NO_2^--N mg $MLVSS^{-1}$ h^{-1}. After 6 days of enrichment, the levels of DEN444- and DEN1454-targeted bacteria decreased (Fig. 2), while the levels of DEN581- and DEN124-targeted bacteria increased to 36% and 47%, respectively, by day 9 (and these bacteria collectively comprised 83% of all

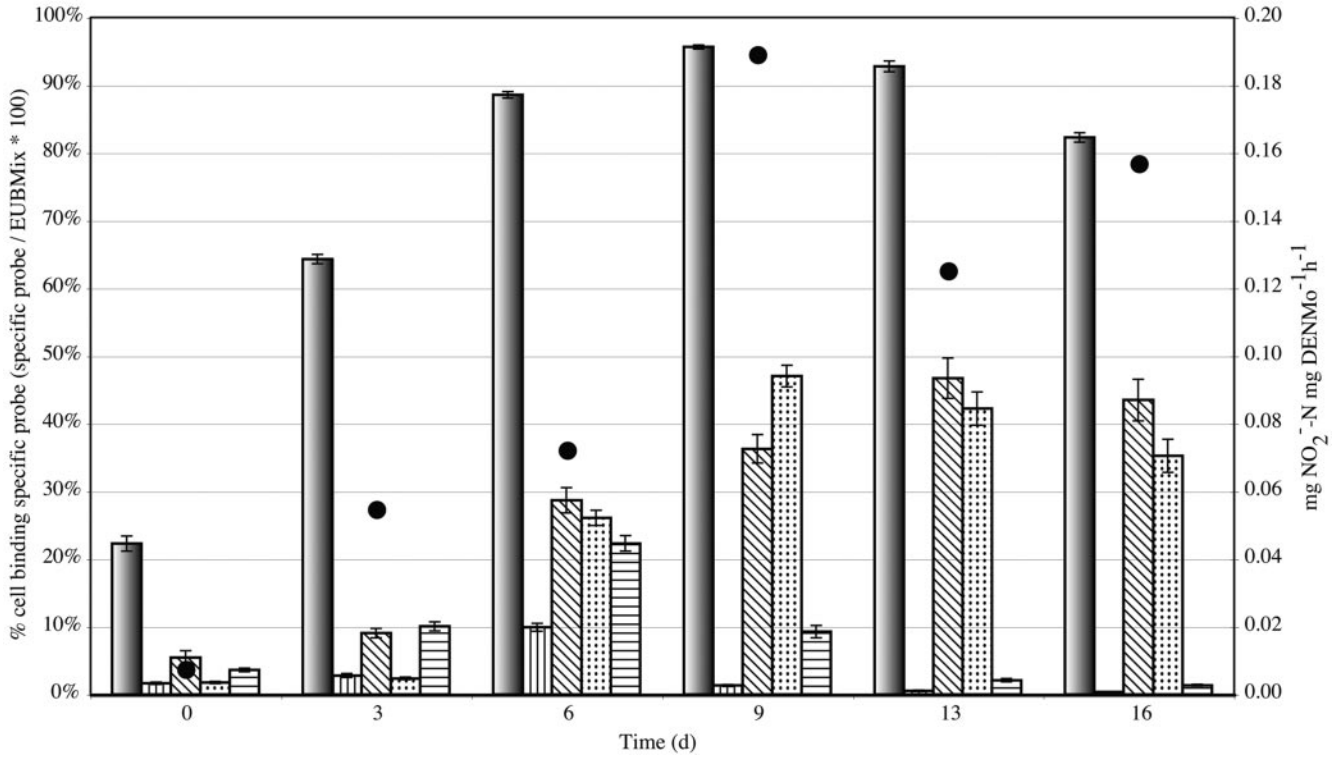

FIG. 2. Bacterial community analysis of CFDSBR sludge as determined by FISH biovolume measurement. The values obtained with the *Betaproteobacteria* probe (shaded bars) and probes DEN444 (bars with vertical lines), DEN581 (bars with diagonal lines), DEN124 (dotted bars), and DEN1454 (bars with horizontal lines) are expressed as percentages of the area of the cells detected with EUBMix. The specific denitrification rate (mg NO_2^--N mg $DENMo^{-1}$ h^{-1}) is indicated for each time analyzed by FISH (DENMo is MLVSS × percentage of total denitrifiers quantified using all four probes).

bacteria) (Fig. 2). The level of *Betaproteobacteria* (BET42a-targeted organisms) increased from 22% to 96% from day 0 to day 9 (Fig. 2).

The cell-specific nitrite consumption rate was calculated for the CFDSBR by assuming that (i) the probe-targeted cells in the biomass were the denitrifiers; (ii) the inert fraction of the MLVSS was negligible or constant throughout operation of the reactor; and (iii) all active microbial cells in the CFDSBR sludge were targeted by the EUBMix probe in the FISH analysis. Figure 2 shows the denitrifier-specific nitrite consumption rates, expressed as mg NO_2^--N mg $DENMo^{-1}$ h^{-1}, for the 16 days of reactor operation (DENMo was defined as MLVSS concentration × percentage of total probe-targeted denitrifiers). NO_2^--N was present only at the end of the cycle on days 0 to 3, and from day 6 on the rate of nitrite consumption was as high as the continuous feeding rate, which resulted in no nitrite accumulation during the entire cycle. However, excess acetate was observed to be present at all times (data not shown), and the COD/NO_2^--N utilization ratio for the enriched acetate-utilizing denitrifiers was calculated based on the cycle study data to be 2.73:1.

There was a significant positive correlation, expressed as Pearson's correlation coefficient (4) ($r = 0.975$, $P < 0.01$), between the abundance of the denitrifying populations identified (the sum of all probe-positive putative acetate-utilizing denitrifying cells) and the increase in denitrification rates measured in the CFDSBR. The fluctuations in denitrification rates observed on days 9, 13, and 16 (Fig. 2) were correlated with slight fluctuations in DEN581- and DEN124-targeted bacteria (particularly for a comparison of day 9 with days 13 and 16), which suggests that there may have been a difference in denitrification capacities between these dominant putative denitrifiers in the reactor. However, when there is constant loading of feed, other factors, such as changes in the MLVSS, also affect the specific denitrification rates.

FISH-MAR with DEN124- and DEN581-targeted organisms. FISH-MAR with the DEN581 and DEN124 probes and [^{14}C]acetate was conducted with the CFDSBR. Figure 3 shows that DEN581-targeted cells took up acetate under anoxic conditions, as demonstrated by the overlap of the yellow cluster in Fig. 3A (DEN581-targeted cells) with the white area in Fig. 3A (silver grains in the autoradiographic film). Similarly, DEN124-targeted cells also took up acetate under anoxic conditions, as shown by the overlap of the yellow cluster in Fig. 3C (DEN124-targeted cells) with the white area in Fig. 3D (silver grains in the autoradiographic film). However, with both probes there were some probe-targeted cells that did not appear to take up radioactively labeled acetate, as indicated by clear (black) areas on the autoradiographic film above the cells on the MAR slides (Fig. 3B and D). Furthermore, in both FISH-MAR experiments, there were some non-probe-targeted cells (green clusters) that appeared to have taken up radioactively labeled acetate, as indicated by white areas outside the

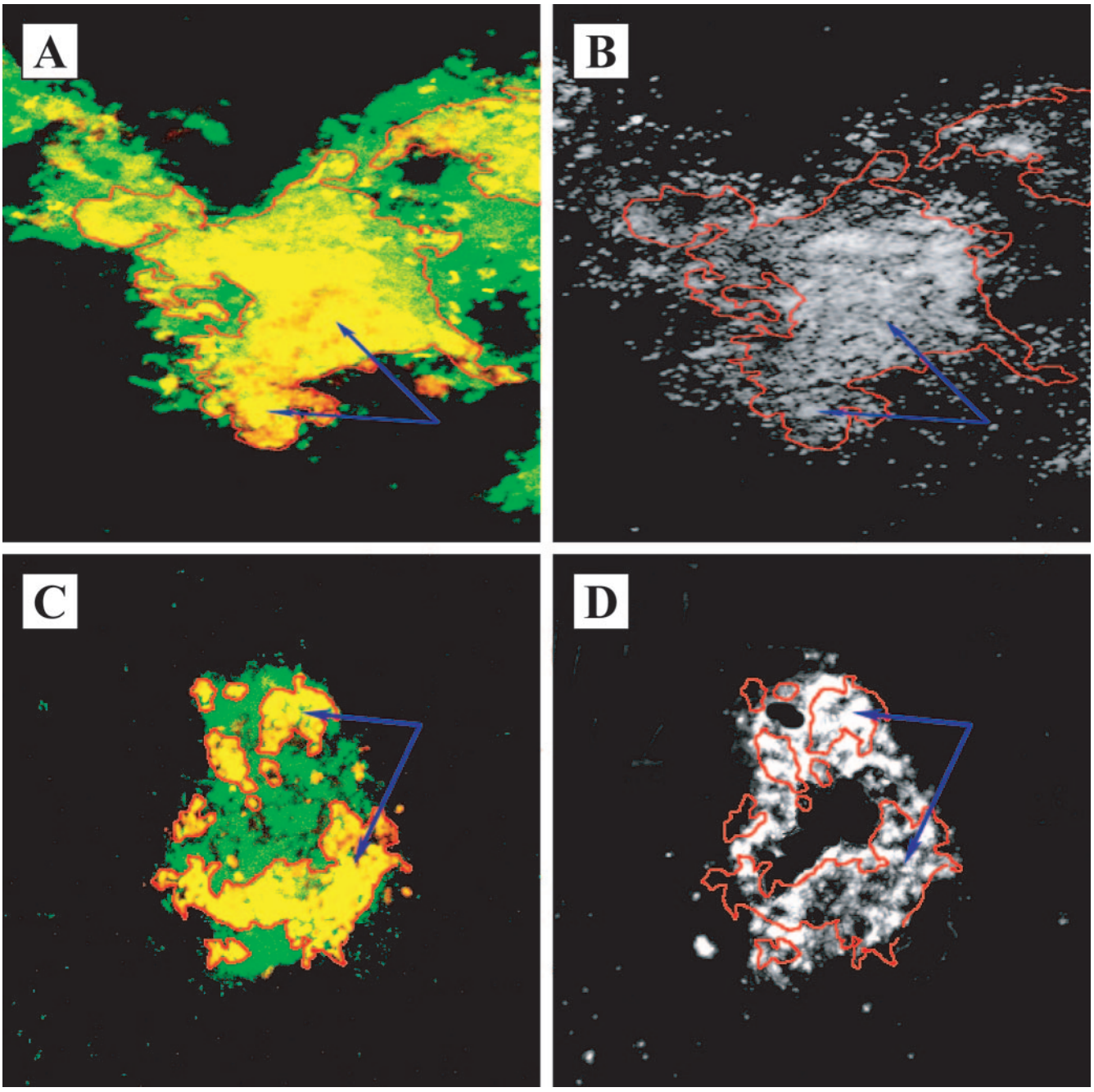

FIG. 3. In situ functional analyses of denitrifying bacteria in the CFDSBR sludge. (A) Confocal laser scanning micrograph of [^{14}C] acetate-fed reactor sludge hybridized with both the EUBMix and DEN581 probes. The outlined yellow cell aggregates and the areas indicated by the arrows are the DEN581-targeted organisms. The image was compiled by obtaining a stack of images by confocal laser scanning microscopy and combining them by orthographic projection into a single image. The image stack was acquired to ensure that all organisms that contributed to the MAR signal (panel B) were visible in the final image. (B) Micrograph of the radiographic film at the same position as the image in panel A. (C) Confocal laser scanning micrograph of [^{14}C]acetate-fed reactor sludge hybridized with both the EUBMix and DEN124 probes. The outlined yellow cell aggregates and the areas indicated by the arrows are the DEN124-targeted organisms. (D) Micrograph of the radiographic film at the same position as the image in panel C.

yellow probe target areas (Fig. 3B and D). This suggests that there were non-probe-targeted bacteria in the sludge that were capable of taking up radioactively labeled acetate under anoxic conditions. The autoradiographic film remained clear in all experiments with pasteurized control biomass and nonpasteurized anaerobic control biomass (data not shown). Hence, the MAR signals obtained with living biomass could not have been caused by adsorption, precipitation, or anaerobic uptake of labeled acetate but must have been the result of active acetate uptake by the cells.

DISCUSSION

SIP with [^{13}C]acetate was directly used to label the DNA of denitrifiers present in full-scale sludge. Although SIP is a largely enrichment-independent method, it is recognized that the incubation step in SIP does provide some organism enrichment. The majority (78%) of the 16S rRNA gene sequences from SIP and cloning analyzed were from the families *Comamonadaceae* and *Rhodocyclaceae* in the *Betaproteobacteria*. Seven oligonucleotide probes were designed to target these 16S rRNA gene sequences, and the in situ levels of the source organisms were determined for full-scale sludge and for enriched CFDSBR biomass. A significant positive correlation between the CFDSBR denitrification rates and the abundance of the probe-targeted denitrifiers was demonstrated. FISH-MAR using the DEN581 and DEN124 probes and [^{14}C]acetate confirmed that probe DEN581- and DEN124-targeted bacteria were capable of taking up acetate under anoxic conditions (Fig. 3). Using a combination of molecular methods, we demonstrated that bacteria related to the genera *Acidovorax*, *Dechloromonas*, and *Thauera* of the families *Comamonadaceae* and *Rhodocyclaceae* are acetate-utilizing denitrifiers in activated sludge.

It was assumed that the experimental approach taken in this study would allow determination and identification of a broader diversity of acetate-utilizing denitrifiers from the source activated sludge than would have been revealed by pure phenotypic enrichment or pure culture isolation. This was likely the case, since the laboratory-scale enrichment (CFDSBR after several days) generated just two dominant acetate-utilizing denitrifiers, while the biomass in the early operational phase of the CFDSBR (like the full-scale sludge) contained at least four relatively abundant denitrifiers.

This study also was one of the first attempts to identify denitrifiers that are able to utilize acetate as a sole source of carbon. In previous research workers have attempted to identify overall denitrifiers present in activated sludge (13, 15, 16, 23, 32, 33). Such research has identified microorganisms belonging to two specific families, the *Comamonadaceae* and the *Rhodocyclaceae*, that are mainly involved in the process of denitrification (15, 16, 32, 33, 41). Even though these findings did not directly focus on acetate-utilizing denitrifiers, it is interesting that clones found in the present study grouped closely, as determined by evolutionary distance analysis, with *Acidovorax* in the family *Comamonadaceae* and *Dechloromonas* and *Thauera* in the family *Rhodocyclaceae*. Juretschko et al. (31) reported that *Thauera* and *Azoarcus* in the family *Rhodocyclaceae* account for as much as 16% of all living bacteria in activated sludge. The probes designed to target denitrifiers in this study confirmed this observation because as many as 12% of the organisms belonging to the two families mentioned above were present in the original seed sludge from the Wacol treatment plant, which had not been exposed to any form of external carbon source augmentation. The findings of Juretschko et al. (31) and our observations suggest that these two families play a major role in denitrification in activated sludge. The occurrence of these organisms in activated sludge also explains the observations made in process studies (30, 35, 38, 43), in which an immediate increase in the denitrification rate was observed when acetate was added.

Previously, workers hypothesized that adaptation and a shift in microbial communities are inevitable when an external carbon source is used for denitrification (26, 30, 35). This is true even with acetate, irrespective of the fact that this compound is a simple source of carbon that can be readily utilized by most microorganisms with a tricarboxylic acid cycle. This study supports the previous observations. Four FISH probe-identified microbial communities dominated the CFDSBR that was seeded with activated sludge. These four denitrifying communities collectively comprised 87% of all bacteria in the reactor within 6 days, indicating that a very rapid shift in the microbial community occurs when acetate is applied to enhance denitrification. Such a dramatic shift, even though it is favorable for denitrification, may affect other biological processes. Therefore, the suitability of adding acetate to a single-sludge wastewater treatment system continuously should be investigated further. The FISH probes designed in this study offer a basis for such a microbial impact assessment.

The Wacol sludge exhibited good settleability, which is important to meet stringent effluent standards in the wastewater industry. In this sludge (the inoculum for the CFDSBR), the DEN probe-targeted bacteria comprised 13% of all EUBmix-targeted bacteria (Fig. 2). However, the sludge that developed in the CFDSBR enrichment exhibited poor settleability. This was associated with an increase in the level of the nonflocculating DEN-targeted bacteria (as shown in Fig. 1) to 87% of all EUBmix-targeted bacteria (Fig. 2). Other research (22) showed that there was a substantial decrease in activated sludge floc sizes when denitrification was facilitated with acetate and increasing nitrate loading rates. Hence, the obvious benefit of using acetate as an external carbon source for denitrification (i.e., high denitrification rates) needs to be balanced against other process performance criteria, like settleability. When the level of DEN probe-targeted bacteria in the sludge biomass (e.g., in full-scale processes) is relatively low, these bacteria do not adversely affect flocculation or settleability.

We used a suite of phenotypic methods (bioreactor studies, SIP) and molecular biological methods to study the in situ abundance and function of acetate-utilizing denitrifiers from wastewater treatment settings. Acetate did facilitate an increase in the denitrification rate (from <0.01 to >0.12 mg NO_2-N mg DENMo^{-1} h^{-1} in 9 days) (Fig. 2) of the mixed microbial community by allowing the level of specific acetate utilizers to increase. In a lab-scale process, enrichment of acetate-utilizing probe DEN124- and DEN581-targeted bacteria was very rapid. The level of these bacteria increased from $<10\%$ to about 80% of all EUBmix-targeted bacteria in 9 days (Fig. 2). However, when acetate was the major carbon source used in denitrification, the settleability of the biomass declined substantially, which was associated with the increase in abundance of the probe DEN124- and DEN581-targeted bacteria. These apparently nonflocculating bacteria are beneficial for denitrification, but they can adversely affect sedimentation when they are abundant.

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